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Filed on 17 September 2002 (17.09.2002)

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
7 October 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/026427 A3

(54) Title: ISOLATION OF IMMUNOGLOBULIN MOLECULES THAT LACK INTER-HEAVY CHAIN DISULFIDE BONDS

(57) Abstract: The current invention features methods for reliably and controllably separating immunoglobulin half antibodies from immunoglobulin whole antibodies, as well as purified immunoglobulin half antibody preparations and purified immunoglobulin whole antibody preparations while preserving biological activity. These dissociated half antibodies can be chromatographically separated from whole antibodies. There are four known subclasses of IgG molecules: IgG_1; IgG_2; IgG_3; and IgG_4. IgG_4 molecules differ from the other IgG isotypes in that the disulfide bonds that link the two heavy chain subunits together do not always form. Due to the non-covalent interactions that hold the heavy chain subunits together, the heterogeneity of IgG_4 molecules is not apparent following gel filtration of purified IgG_4 protein. However, when purified IgG_4 protein is separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions, two distinct protein species can be identified - whole antibody and "half-antibodies".

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/28543

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/395; C07K 16/02, 16/04, 16/06; C12P 21/08; G01N 33/53, 33/531, 33/563
US CL : 435/7.1, 70.21, 328; 530/387.1, 387.3, 389.1, 412, 413, 415, 416, 417

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1, 70.21, 328; 530/387.1, 387.3, 389.1, 412, 413, 415, 416, 417

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,479,895 B1 (AUDITORE-HARGREAVES) 30 October 1984 (30.10.1984), see entire document.	1-62
Y	US 5,292,668 B1 (PAULUS) 08 March 1994 (08.03.1994), see entire document.	1-62
Y	US 6,329,507 B1 (MEZES et al.) 11 December 2001 (11.12.2001), see entire document.	1-62
Y	KRETZSCHMAR et al. High-Level Expression in Insect Cells and Purification of Secreted Monomeric Single-Chain Fv Antibodies. Journal of Immunological Methods. 1996, Vol. 195, pages 93-101, see entire document.	1-62

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
26 June 2004 (26.06.2004)

Date of mailing of the international search report
28 JUL 2004

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US03/28543

Continuation of B. FIELDS SEARCHED Item 3:
EAST
Terms: monovalent, half, antibody, immunoglobulin, Fv, purif?

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

60744.70028WO 00

PCT

WRITTEN OPINION

(PCT Rule 66)

To:
OLSEN V. BYRON
175 CROSSING BLVD., SUITE 410
FRAMINGHAM, MA 01702

Date of Mailing *(day/month/year)*	14 AUG 2007
Applicant's or agent's file reference GTC-56 PCT	REPLY DUE within 1 months/days from the above date of mailing

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US03/28543	11 September 2003 (11.09.2003)	17 September 2002 (17.09.2002)

International Patent Classification (IPC) or both national classification and IPC

IPC: Please See Continuation Sheet
USPC: 435/7.1,70.21,328;530/387.1,387.3,389.1,412,415,416

Applicant

GTC BIOTHERAPEUTICAS, INC.

1. This written opinion is the first (first, etc,) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	☒	Basis of the opinion
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☐	Lack of unity of invention
V	☒	Reasoned statement under Rule 66.2 (a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	☐	Certain documents cited
VII	☐	Certain defects in the international application
VIII	☒	Certain observations on the international application

3. The applicant is hereby **invited to reply** to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension. See rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 17 January 2005 (17.01.2005).

Name and mailing address of the IPEA/US	Authorized officer
Mail Stop PCT, Attn: IPEA/ US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	James L. Grun Telephone No. (571) 272.1600

Form PCT/IPEA/408 (cover sheet)(July 1998)

RECEIVED
Wolf, Greenfield & Sacks, P.C.
AUG 27 2007
Docketed ✓ Already Docketed ____
Atty Required

WRITTEN OPINION

International application No.
PCT/US03/28543

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed

☒ the description:
pages 1-32, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the claims:
pages 33-38, as originally filed
pages NONE, as amended (together with any statement) under Article 19
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the drawings:
pages 1-17, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

☒ the sequence listing part of the description:
pages NONE, as originally filed
pages NONE, filed with the demand
pages NONE, filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE _____

☒ the claims, Nos. NONE _____

☒ the drawings, sheets/~~fig~~ NONE _____

5. ☐ This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed."*

WRITTEN OPINION

International application No.
PCT/US03/28543

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	6, 7, 16-19, 31-44 and 56-62	YES
	Claims	1-5, 8-15, 20-30 and 45-55	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-62	NO
Industrial Applicability (IA)	Claims	1-62	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS
Please See Continuation Sheet

WRITTEN OPINION

International application No.

PCT/US03/28543

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 58 and 62 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): The claims do not further limit the subject matter of the prior claim from they depend because a HIC column does not further limit the prior claimed ion exchange column.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

TIME LIMIT:
The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

Continuation of IPC:
A61K 39/395(2006.01);**C07K 16/04**(2006.01),**16/06**(2006.01);**G01N 33/531**(2006.01),**33/563**(2006.01);**C12P 21/08**(2006.01)

V. 2. Citations and Explanations:
Claims 1-62 meet the criteria set out in PCT Article 33(4), and thus have industrial applicability because the subject matter claimed can be made or used in industry.

Claims 1, 2, 5, 8, 9, 12, 20, 22, 24-27, 29, 30, 45, 47-50 and 52-55 lack novelty under PCT Article 33(2) as being anticipated by **PALMER** et al. (Biochem. 3: 863, 1964).

PALMER et al. reduced rabbit IgG prepared from serum samples to produce a proportion of half-IgG molecules in the preparations, the pH was reduced to dissociate the non-covalent interactions of the half-IgG molecules, and the reduced and lowered pH sample was applied to a column to separate the half-IgG and whole IgG molecules (see e.g., Fig. 3).

Claims 1-4, 10, 12-15, 20-30 and 45-55 lack novelty under PCT Article 33(2) as being anticipated by **KING** et al., (Biochem. J. 281: 317, 1992).

KING et al. reduced the pH of mixtures containing IgG4 half (including preparations of Fab') and whole (including $F(ab')_2$) chimeric or myeloma antibodies and applied the mixtures to series of columns including ion exchange columns. The method involved lowering of the pH with a linear pH gradient to levels capable of dissociating non-covalently bound immunoglobulins. The eluted material was further separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, including with a rod (i.e. columnar) gel.

Claims 1, 2, 5, 8, 11, 12, 15, 20, 22, 24-27, 29, 30, 45, 47-50 and 52-55 lack novelty under PCT Article 33(2) as being anticipated by **PAULUS** (US 5,292,668).

PAULUS lowered the pH of a mixture of Fab' monomers and $F(ab')_2$ IgGI antibodies and separated the populations on a chromatography column (see e.g., cols. 7-9).

Claims 1-62 lack an inventive step under PCT Article 33(3) as being obvious over the combined teaching of **KING** et al. (Biochem J. 281: 317, 1992), **SCHUURMAN** et al (Molecular Immunol. 38: 1, 2001), **ANGAL** et al (Mol. Immunol. 30:

WRITTEN OPINION

International application No.
PCT/US03/28543

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

105, 1993), and Palmer et al. (Biochem. 3: 863, 1964).

King et al. teach mixtures containing IgG4 half (including preparations of Fab') and whole (including $F(ab')_2$) chimeric or myeloma antibodies and applied the mixtures to series of columns including ion exchange columns. The reference teaches the desirability of separating the half from the whole antibodies for further studies of the hinge region of the molecules (see e.g. pages 321-322), but did not separate the molecules other than by sodium dodecyl sulfate polyacrylamide gel electrophoresis, including with a rod (i.e. columnar) gel.

Schuurman et al. teach the equilibrium of half and whole human IgG4 antibodies and also teach IgG4 hinge mutants with reduced ability to form half antibody molecules. The reference suggests that the non-covalent interactions of half antibodies, particularly the interactions between the C_H3 domains, can be dissociated by denaturing conditions such as low pH (see e.g. page 6).

Palmer et al. teach dissociation of the non-covalent interactions of half-IgG molecules by low pH and size exclusion chromatography for the separation of dissociated half from whole IgG.

Angal et al. teach the chimeric antibody of King et al. having a further mutation in the hinge region to a sequence similar to that found in IgG1 and IgG2, a mutation which essentially abolishes the half IgG4 antibody molecules in the preparations. The reference suggests partial resolution of the non-mutated half and whole antibodies by ion exchange chromatography, but does not provide details therefor (see page 105).

It would have been obvious to one of skill in the art to have separated half and whole antibodies, particularly human or chimeric IgG4 as suggested in King et al., with a reasonable expectation of success by reducing the pH of a sample prior to a column separation, because a reduction in pH is directly suggested by Schuurman et al. or Palmer et al. for the dissociation of non-covalently associated half antibodies, specifically prior to a column separation (Palmer et al.). One would have reasonably expected any of size exclusion (Palmer et al.) or ion exchange (Angal et al.) or other, such as hydrophobic interaction, chromatography to have performed the separation because these were known to the art to function for the separation of antibodies, some specifically for the separation of dissociated half from whole antibodies. One would have reasonably expected that the source of the antibody would not have affected the presence of a mixture because the ability to form half antibodies is a property of some antibody isotypes (King et al., Schuurman et al., Angal et al.) or of some treatments (Palmer et al.) and one would have reasonably expected that the source of the antibody mixture would not have affected the downstream separation.

---------- NEW CITATIONS ----------

PALMER et al. Dissociation of Rabbit Gamma-Globulin into Half-Molecules after Reduction of One Labile Disulfide Bond. Biochemistry. June 1964, Vol. 3, No. 6, pages 863-869, see entire document.

KING et al. Expression, Purification and Characterization of a Mouse-Human Chimeric Antibody and Chimeric Fab' Fragment. Biochemical Journal. 1992, Vol. 281, pages 317-323, see entire document.

ANGAL et al. A Single Amino Acid Substitution Abolishes the Heterogeneity of Chimeric Mouse/Human (IgG4) Antibody. Molecular Immunology. 1993, Vol. 30, No. 1, pages 105-108, see entire document.

SCHUURMAN et al. The Inter-Heavy Chain Disulfide Bonds of IgG4 are in Equilibrium with Intra-Chain Disulfide Bonds. Molecular Immunology. 2001, Vol. 38, pages 1-8, see entire document.

COLCHER et al. Characterization and Biodistribution of Recombinant and Recombinant/Chimeric Constructs of Monoclonal Antibody B72.3. Cancer Research. 01 April 1989, Vol. 49, pages 1738-1745, see entire document.